

Actin, Microvilli, and the Fertilization Cone of Sea Urchin Eggs

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ABSTRACT Sea urchin eggs and oocytes at the germinal vesicle stage were fixed at various times after insemination, and thin sections were examined. Actin filaments can first be found in the cortical cytoplasm 1 min after insemination, and by 2 min enormous numbers of filaments are present. At these early stages, the filaments are only occasionally organized into bundles, but one end of many filaments contacts the plasma membrane. By 3 min, and even more dramatically by 5 min after insemination, the filaments become progressively more often found in bundles that lie parallel to the long axis of the microvilli and the fertilization cones. By 7 min, the bundles of filaments in the cone are maximally pronounced, with virtually all the filaments lying parallel to one another. Decoration of the filaments with subfragment 1 of myosin shows that, in both the microvilli and the cones, the filaments are unidirectionally polarized with the arrowheads pointing towards the cell center. The efflux of H^+ from the eggs was measured as a function of time after insemination. The rapid phase of H^+ efflux occurs at the same time as actin polymerization. From these results it appears that the formation of bundles of actin filaments in microvilli and in cones is a two-step process, involving actin polymerization to form filaments, randomly oriented but in most cases having one end in contact with the plasma membrane, followed by the zippering together of the filaments by macromolecular bridges.

Although actin is a major constituent of all eukaryotic cells, the controls over its assembly into filaments and/or the packing of these filaments into bundles are still poorly understood. One of the most extraordinary examples of changes in actin occurs in sea urchin eggs after fertilization. Before fertilization, few, if any, actin filaments are present, yet a few minutes after fertilization microvilli with actin filaments within them have sprouted all over the surface of the egg (3, 21). Even more spectacular is the so-called fertilization cone, a protoplasmic extension that forms, on many species of marine eggs, at the point of contact between the acrosomal process of the sperm and the egg. Microfilaments have been found in the cone (12–14, 26), but the chemical nature of these filaments and their exact location in the cone have not been reported, probably because of severe difficulties encountered in fixing actin filaments in cells (16).

Literature on the fertilization cone began with classic papers by Fol in 1877 (6–8). He described the cone in sea urchin and starfish eggs. In the latter, the sperm undergo the acrosomal reaction when they come in contact with the thick layer of jelly that surrounds the egg; this takes place 15 μ m from the egg

plasma membrane. What Fol saw and dutifully described was a mound of cytoplasm that formed on the surface of the egg beneath the sperm. However, he could not see a connection between the sperm and the egg, probably because his microscope was inadequate to resolve the acrosomal process, which measures only 0.05 μ m in diameter. Nevertheless, the sperm moved towards this mound and accordingly Fol called it the “cone d’attraction,” a concept that made him infamous—after all, how did the egg know where the sperm was, 15 μ m away? In a delightful series of papers, Fol rebuffed all criticism (6, 7). It was not until 1930, some 50 years later, that the matter was resolved by Chambers (4), who showed that the cone is continuous with the sperm via the acrosomal process.

Perhaps because of this inauspicious beginning, studies on the fertilization cone have been rare, a surprising fact in view of the voluminous literature on fertilization in echinoderms. This is a pity because the fertilization cone, it seems to us, is a magnificent experimental object. It forms on the surface of a large cell that can be penetrated by microelectrodes. It moves with ameboid motion, a fact first established by Seifriz (19) in 1926. It can be huge, exceeding 35 μ m in length and 15 μ m in

diameter and, as will be shown in this report, is full of polarized actin filaments. It is an object that we are hopeful will be amenable to experiments on questions such as are fluxes of ions or a change in resting potential essential for actin assembly and actin bundle formation, how does a cell locally change its intracellular environment to induce movement and filament assembly, and what do differences in the cones that form at varying stages in development (11, 12, 19) tell one about differences in the chemical composition of the cytoplasm.

The present paper will describe how actin filaments assemble and are patterned in microvilli and in the fertilization cone of mature and immature eggs, and will relate these changes to proton efflux from the egg. What we find suggests that in the formation of both microvilli and the cone, the actin filaments become patterned in a two-step process: actin assembly, possibly from the plasma membrane, is followed by a zipper together of actin filaments.

MATERIALS AND METHODS

Obtaining Organisms and Gametes

Arbacia punctulata were obtained from the supply department of the Marine Biological Laboratory at Woods Hole, Mass., and from the Gulf Specimen Co., Panama, Fla. Mature eggs and sperm were obtained by injecting 500 mM KCl; fertilization was accomplished by mixing eggs and sperm at 20°C at final dilutions of 1:200 and 1:1,000, respectively. Oocytes at the germinal vesicle stage (GV) were obtained from organisms that were not completely ripe. The ovaries were suspended in seawater, which allowed the release of eggs and GV oocytes. The GV oocytes were isolated with pipettes and put in small dishes of seawater.

Examination of Living Material

Fertilization and the formation and movement of fertilization cones were examined with a Zeiss phase-contrast microscope, with an oil immersion objective. Movies were taken by attaching a 16-mm Bolex camera to the microscope via a homemade stand. Best results were obtained by taking the movies at 2 frames/s with an electric clock motor coupled to the movie camera.

Fixation of Eggs and GV Oocytes for Electron Microscopy

Several methods were used. We will list these below and indicate in the figure legends which method applies to each figure. *Arbacia punctulata* is the only species with which we could obtain reasonable fixation repeatedly; eggs of *Strongylocentrotus purpuratus*, *Paracentrotus lividus*, and *Urechis caupo* were not well fixed by these procedures.

PROCEDURE 1: Eggs or GV oocytes were immersed in 1% glutaraldehyde (obtained as an 8% solution from Electron Microscope Sciences, Fort Washington, Pa.) in seawater (1 ml of glutaraldehyde solution to 7 ml of seawater) for 30 min at room temperature. The specimens were washed briefly in seawater and postfixed in 1% OsO_4 in 100 mM phosphate buffer at pH 6.0 at 0°C for 30 min. The specimens were then washed three times in distilled water at 0°C and stained *en bloc* in 0.5% uranyl acetate in water at 0°C for 3 h or overnight, then dehydrated rapidly in acetone and embedded in Araldite.

PROCEDURE 2: Before fertilization, the eggs were preincubated for 5 min in seawater with 0.1% nicotine sulfate and 10 mM Tris at pH 8.0 at 21°C. Nicotine induces polyspermy (15). Sperm were then added and the fertilized eggs were fixed at various times by the addition of glutaraldehyde to make a 1% solution in the seawater. Fixation, washing, postfixation, *en bloc* staining, and dehydration were carried out as in procedure 1.

PROCEDURE 3: Eggs were preincubated with nicotine sulfate in Tris seawater, then fertilized and fixed with 1% glutaraldehyde seawater as in procedure 2. 30 min after the addition of glutaraldehyde, the fertilized eggs were washed three times with a solution containing 100 mM PIPES buffer at pH 7.0, 475 mM NaCl, and 25 mM KCl. The eggs were then incubated in 1% tannic acid (Mallinkrodt Inc., St. Louis, Mo.) in 100 mM phosphate buffer at pH 7.0 for 15 min at 21°C, washed in 100 mM phosphate buffer, and postfixed in 1% OsO_4 in 100 mM phosphate buffer at pH 6.0 for 30 min at 0°C. The eggs were then rinsed three times in water and *en bloc* stained in 0.5% uranyl acetate for 3 h, dehydrated in acetone, and embedded.

PROCEDURE 4: To study decoration with subfragment 1 of myosin (S_1), we preincubated and fertilized eggs in seawater containing 0.1% nicotine sulfate. 10

mM Tris at pH 8.0, and 4 mM Na_2SO_4 . Na_2SO_4 prevents the fertilization membrane from becoming impermeant to large molecular weight substances (27) and thus allows the S_1 to pass through it. 5 min after insemination, the plasma membranes of the eggs were broken by suspending the eggs in a hypotonic medium of 5 mM MgCl_2 , 10 mM EGTA, 50 mM phosphate buffer at pH 6.9, 0.5 mg/ml phenylmethyl sulfonyl fluoride (PMSF), and 0.1 mg/ml soybean trypsin inhibitor. After 2 min, the eggs were resuspended in the same medium containing 3 mg/ml S_1 . The S_1 was kindly prepared and donated by Dr. Annemarie Weber in the Department of Biochemistry, University of Pennsylvania, Philadelphia, Pa. Incubation in S_1 proceeded for 30 min at 21°C. The excess buffer and S_1 were aspirated off and the eggs were washed in the above solution lacking S_1 . This solution was removed and the eggs fixed for 30 min at 21°C in 1% glutaraldehyde in 50 mM phosphate buffer at pH 6.9, containing 0.2% tannic acid (2). They were washed in 100 mM phosphate buffer and postfixed in 1% OsO_4 in 100 mM phosphate buffer at pH 6.0 on ice for 45 min. The eggs were then washed in water three times, *en bloc* stained, dehydrated, and embedded.

Electron Microscopy

Thin sections were cut with a diamond knife on a Sorvall Porter-Blum ultramicrotome (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del.), stained with uranyl acetate and lead citrate, and viewed with a Philips 200 electron microscope.

Measurement of Acid Efflux

Acid efflux at fertilization was measured by continuously titrating the egg suspension to pH 8.0, and measuring the time-course and amount of NaOH added (17). 10 ml of eggs (35,000 eggs/ml, jelly present) was stirred slowly and titrated with a Radiometer pH stat (Copenhagen, Denmark). Sperm were added to a final dilution of 1:2,000 or 1:10,000; nicotine was not present in the seawater. Under the conditions used, 40 s were required to return the pH to 8.0 after an instantaneous addition of an amount of acid comparable to that produced by the egg sample at fertilization; however, the response started with a delay of <1 s after addition of the acid, and proceeded linearly over the 40-s period. Consequently, although we can correctly measure the delay between insemination and the beginning of increased acid release by eggs, the shape of the time-course of release may be slightly distorted.

RESULTS

Light Microscope Observations on the Formation and Movements of Fertilization Cones

MATURE EGGS: Cones on mature eggs generally do not exceed 1–5 μm in height. In 1- μm sections of fertilized eggs, they appear as small granule-free masses of cytoplasm that are often very irregular in form (Fig. 1).

GV OOCYTES: The cones of GV oocytes are obvious even at low magnification in the light microscope (Figs. 2 and 3) as was first observed by Seifriz in 1926 (19). They can exceed 35 μm in length and 15 μm in diameter (Fig. 3), and are visible by 2 min after insemination. They are free of yolk granules and mitochondria (Fig. 2). They undergo rapid movements resembling those of an ameba with its tail stuck fast to the surface of the egg (Fig. 4). Pseudopods form from the surface of the cone and then move, first in one direction, then another, as seen in the movie sequence (Fig. 4). New pseudopods can form and begin to stream within 10 s.

Fine-structure Observations on the Fertilization Cones and Microvilli of Mature Eggs

When eggs are fixed before the addition of sperm or within one min afterwards, we see along the surface, in regions where the cortical reaction has not yet occurred, numerous tiny bumps, microvilli (1), that do not exceed 0.1–0.12 μm in length in *Arbacia* (Fig. 5). Beneath the plasma membrane is a meshwork of a finely fibrillar material, but clearly defined filaments are not seen. Surrounding the outer surface of the plasma

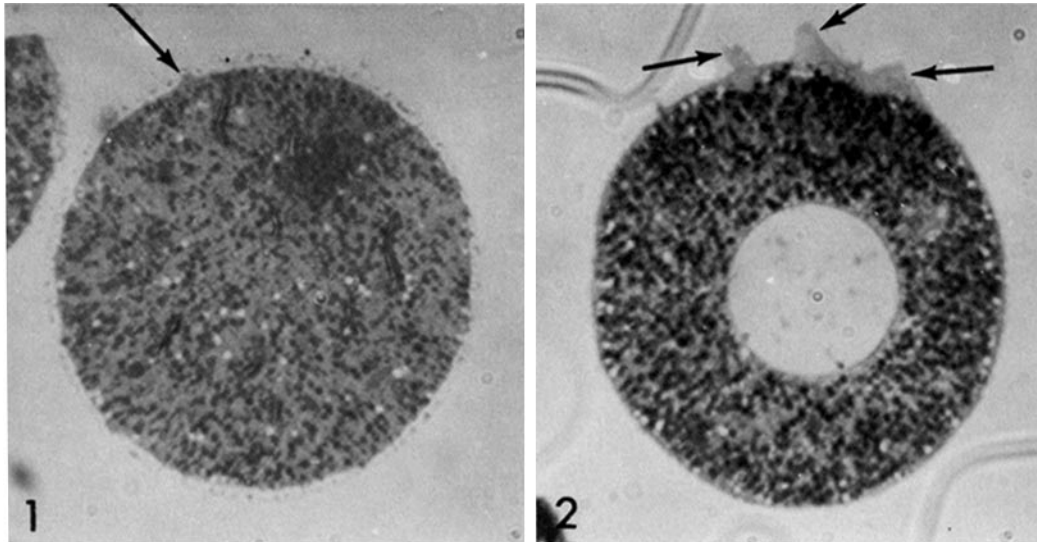


FIGURE 1 Plastic section through an *Arbacia* egg fixed 5 min after insemination. The arrow indicates a typical fertilization cone. $\times 640$.

FIGURE 2 Plastic section through a GV *Arbacia* oocyte fixed 5 min after insemination. The arrows indicate three cones. Note that they are granule free and much larger than those of a mature egg. $\times 640$.

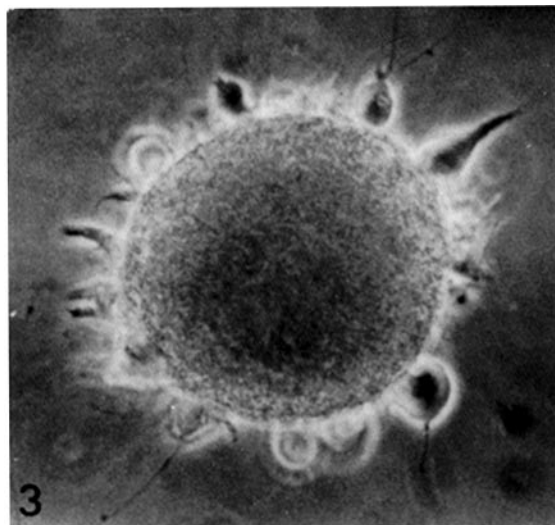


FIGURE 3 Light micrograph of a living GV *Arbacia* oocyte taken ~ 7 min after insemination. Note the number and size of the cones extending from this oocyte. The sperm flagellum can be seen extending from some of the cones. $\times 500$.

membrane is a thin layer of an amorphous material, the vitelline layer.

In this and most of the following preparations, the eggs were preincubated and inseminated in nicotine, to induce polyspermy (15), a state that increases the chances of cutting sections through cones. We have compared eggs fixed at 1 and 2 min after being fertilized with or without pretreatment in nicotine, and, except for a slight delay in the cortical reaction in the nicotine samples, no differences were found. Longo and Anderson (15) also reported that nicotine has little effect on the morphology and kinetics of the cortical reaction and fertilization cone formation. In eggs fixed 1 min after the addition of sperm, cortical granule exocytosis is complete in some; in others, and in particular in those incubated with nicotine (procedure 2), exocytosis has occurred over only part of the

surface of the egg. In that part of the egg where exocytosis has taken place, we find irregular surface projections containing filaments. In general, the number of filaments is small and they do not parallel each other to form bundles. In regions where exocytosis has not yet occurred, we do not see filaments.

In contrast to the image of the cortex seen 1 min after insemination, by 2 min, surface projections up to $0.8 \mu\text{m}$ in length yet very irregular in form can be found all over the surface of the egg (Fig. 6). Within these cell extensions we find filaments; in general, most of the filaments are not parallel to each other but appear randomly arranged. One end of many of the filaments appears to terminate in association with the plasma membrane (Fig. 6). Although electron-dense spots are sometimes seen along the cytoplasmic surface of the plasma membrane, there is no clear association between the densities and the filaments like that seen in intestinal brush-border microvilli (24). Transverse sections through the projections show that if filaments associate to form bundles (e.g., the left side of Fig. 6), we can count 10–20 filaments in a bundle. Sections through fertilization cones are also commonly encountered. In favorable sections, a portion of the sperm head and/or flagellar axoneme can be located within the cone, but this is not necessary to identify a cone, which appears in sections as a large, irregularly-shaped process lacking mitochondria, yolk particles, Golgi bodies, and vesicles. Instead, each cone contains large numbers of ribosomes and filaments. The filaments are most often randomly arranged in the cone. In some cones we find two or three bundles of filaments. These bundles, in general, are oriented perpendicular to the egg surface, and in transverse sections we counted ~ 10 –30 filaments in each bundle.

By 3 min after insemination, irregular surface projections are still common, but, by now, slender cell extensions or microvilli can be found. Most frequently these microvilli extend from the irregular surface projections; often multiple microvilli extend from one projection (Fig. 7c). In longitudinal sections, we find a bundle of parallel filaments within each microvillus (Fig. 7c). In transverse sections, it is possible to count the

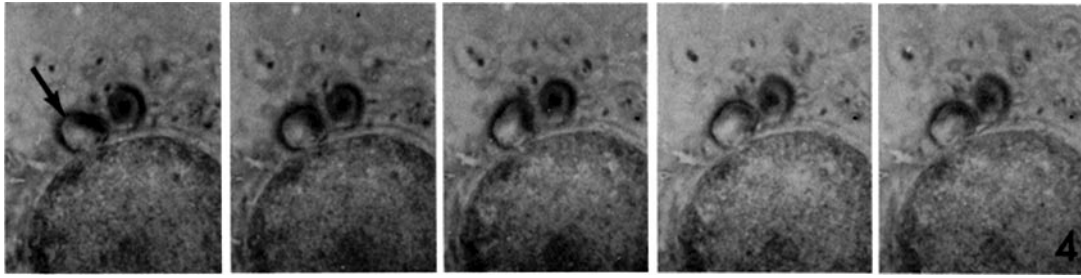


FIGURE 4 Frames from a film illustrating the movement of the cone indicated with the arrow on an oocyte of *Arbacia*. These frames were taken at 5-s intervals. $\times 375$.

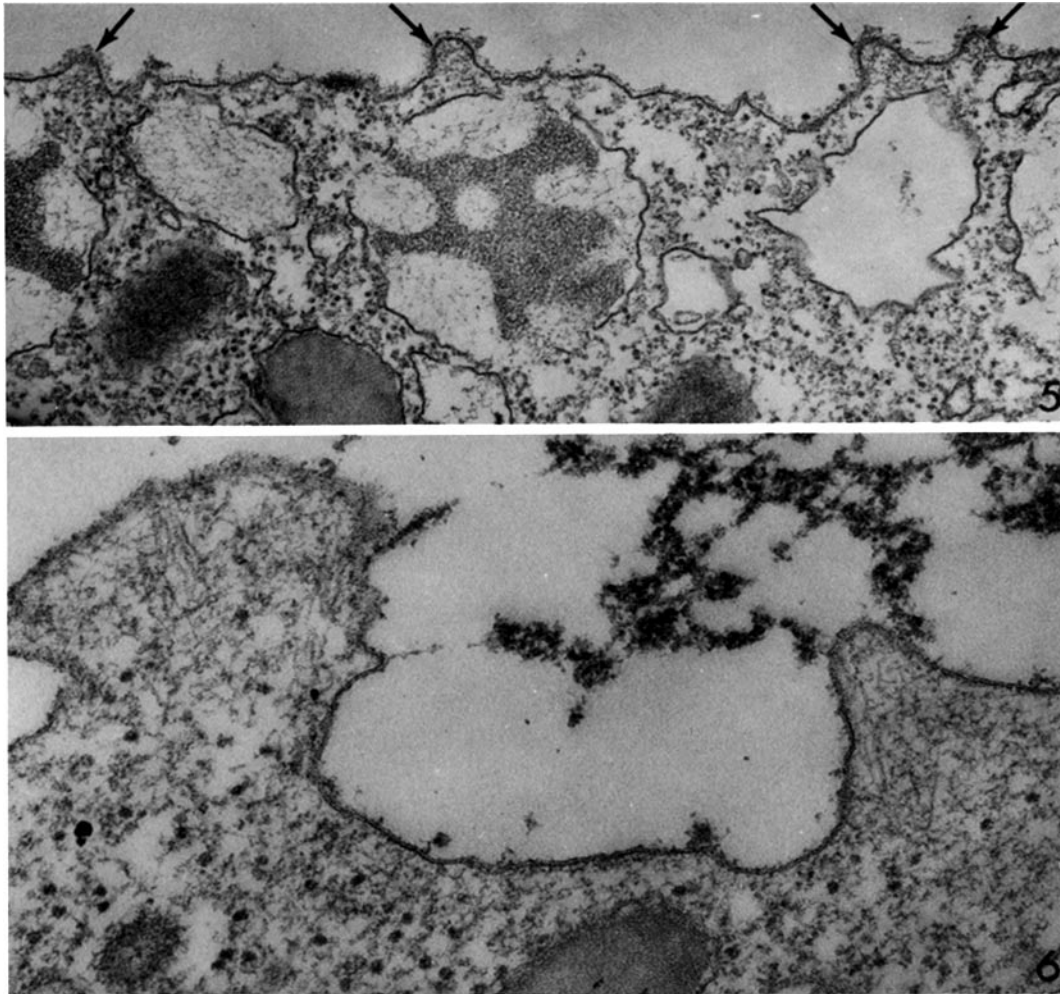


FIGURE 5 Thin section through a portion of an *Arbacia* egg that has not yet undergone the cortical reaction. The egg was fixed by procedure 2, 1 min after insemination. The arrows indicate the tiny microvilli at this stage, within which is an amorphous material. $\times 60,000$.

FIGURE 6 Portion of the cortex of an *Arbacia* egg fixed by procedure 3, 2 min after insemination. Of interest are the two protrusions within which are filaments. $\times 102,000$.

number of filaments in each bundle; we count between 12 and 40 (Fig. 7 *d*). Around these bundles is a population of randomly arranged filaments (Fig. 7 *b-d*). Particularly interesting is that in many cases one end of the filament appears to make contact with the plasma membrane (Fig. 7 *a-c*). From the images presented in Fig. 7 and many others, it appears that the filaments assemble first with random orientation, yet with one end contacting the plasma membrane, and then gradually associate together to form bundles. A possible sequence in this

association is illustrated in Fig. 7 *a-c*. The fertilization cones by 3 min after insemination resemble those after 2 min. Within these cones we often see a portion of the flagellar axoneme. Numerous filaments are present. Many of the filaments are randomly oriented, although a few loose bundles of filaments are visible (Fig. 8).

By 5 min after insemination, long, straight, more conventional-looking microvilli up to $1.4 \mu\text{m}$ in length can be seen extending from the surface of the egg (Fig. 9 *b*). Within each

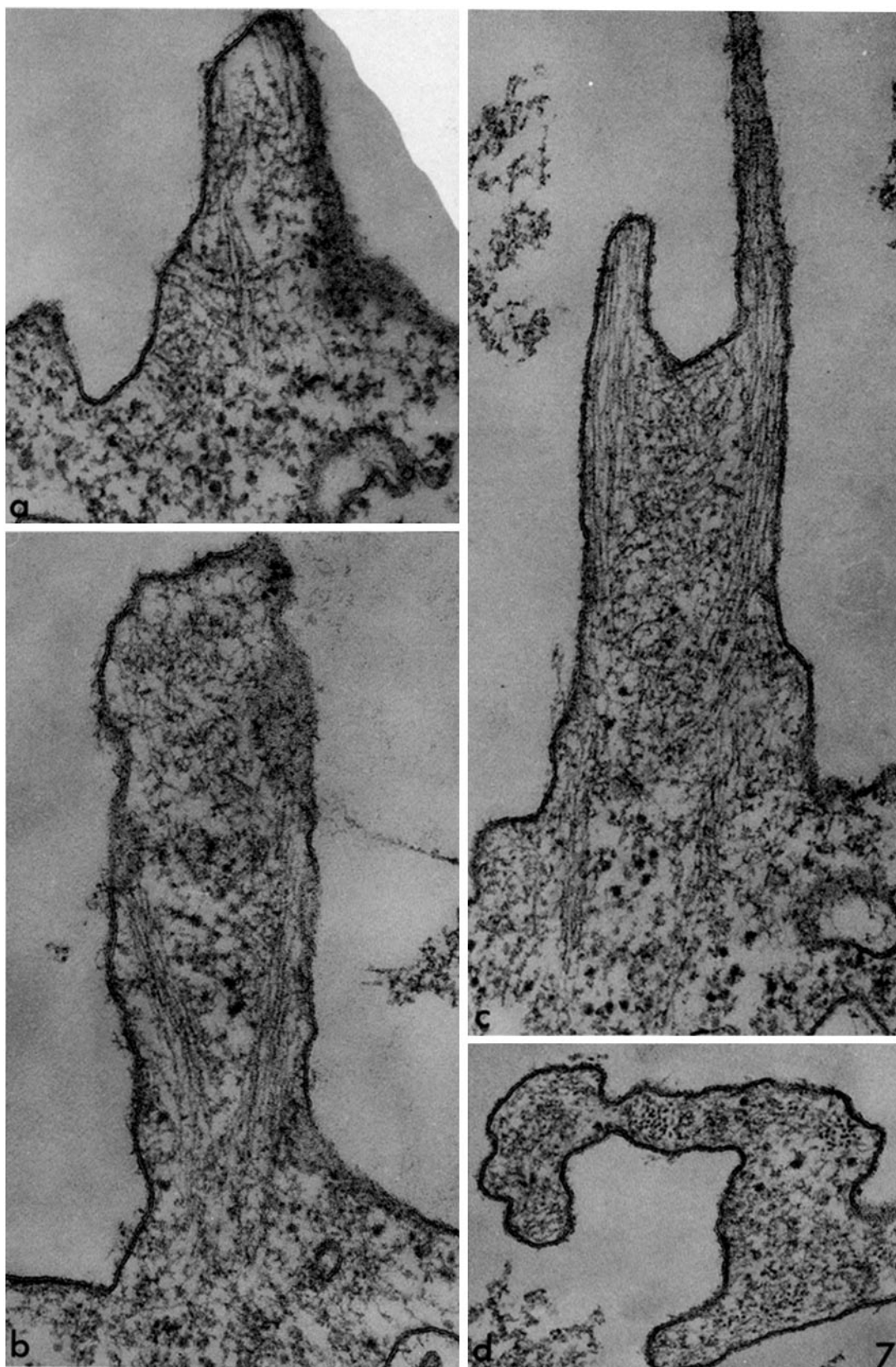


FIGURE 7 Portions of the cortex of an *Arbacia* egg fixed by procedure 2, 3 min after insemination. *a*–*c* depict what we suggest might be stages in the assembly of microvilli. Of interest is that many of the filaments appear to contact the limiting membrane. *d* is a transverse section through a process similar to that depicted in *c*. *a*, $\times 81,000$; *b*, $\times 62,000$; *c*, $\times 106,000$; *d*, $\times 106,000$.

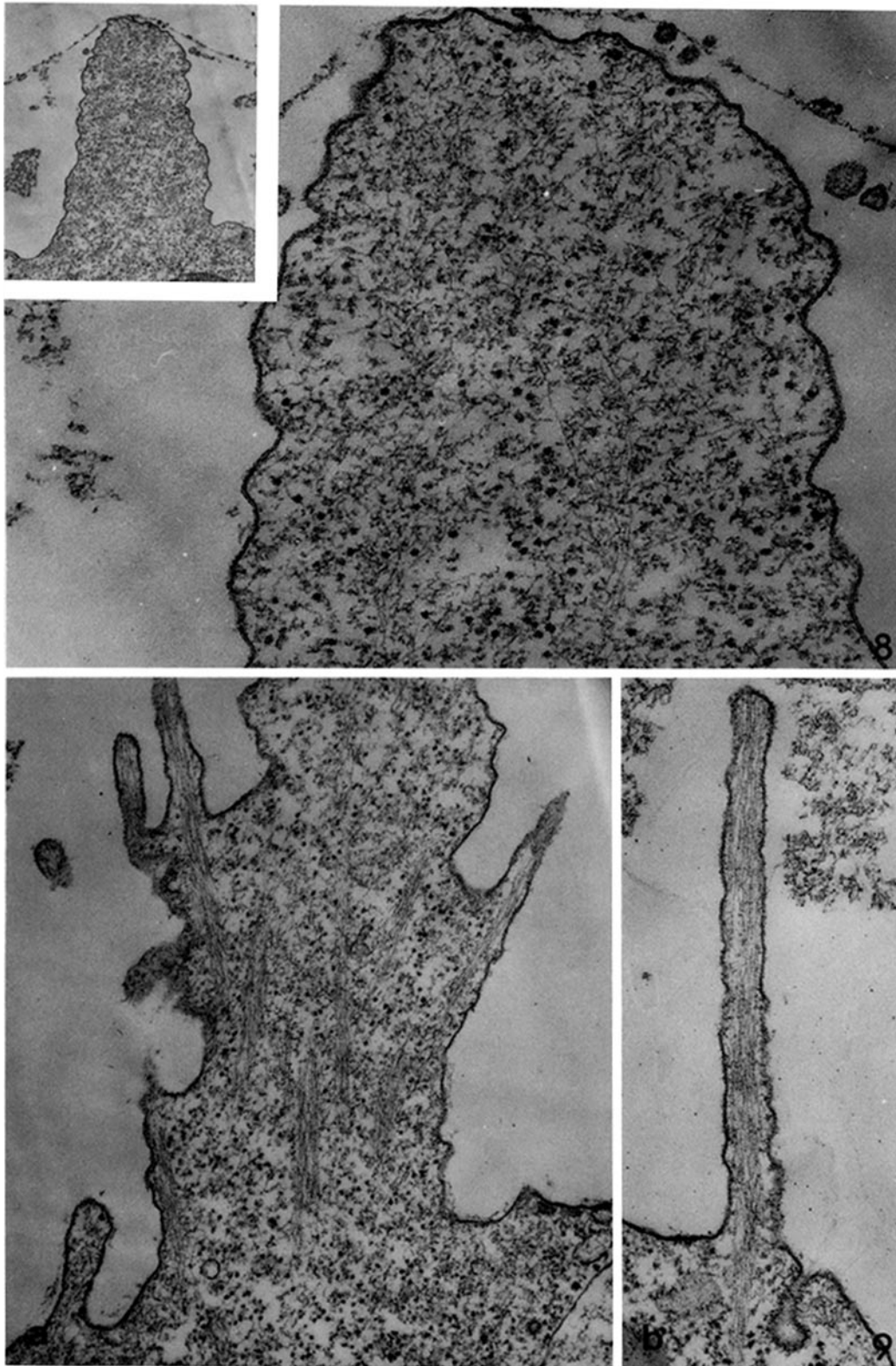


FIGURE 8 Thin section through a fertilization cone from an *Arbacia* egg fixed by procedure 2, 3 min after insemination. The *inset* shows the cone at low magnification ($\times 17,500$); the rest of the figure shows the cone at higher magnification ($\times 84,500$). Of interest is the presence of numerous filaments in the cone, many randomly oriented, some forming loose bundles.

FIGURE 9 (a) Thin section through a portion of the fertilization cone from an *Arbacia* egg fixed by procedure 2, 5 min after insemination. Note that the cone presents a spikelike profile rather than a smooth contour. Within each spike is a bundle of actin filaments. $\times 46,000$. (b) Thin section through a microvillus that extends from the surface of an *Arbacia* egg fixed by procedure 2, 5 min after insemination. $\times 67,000$.

microvillus is a bundle of filaments, which can often be seen to extend into the cortical cytoplasm. There are still many places on the egg surface from which irregular cell extensions project, but unlike preceding stages, at this stage long, straight, unbranched microvilli are not an exception but are common. The fertilization cone has also changed remarkably by this time. The smooth-surfaced cone that was present in the earlier stages has been replaced by a cone with many spikelike processes (Fig. 9a) resembling what Seifriz (19) referred to as a "flame-like process." Within each spike is a bundle of filaments. Bundles of filaments often extend from the cone into the cortical cytoplasm, where they are sometimes found surrounding the axoneme. The impressive feature of the cones at this stage is the enormous number of filaments involved and their being almost all in bundles rather than existing as isolated filaments. For example, in the longitudinal section illustrated in Fig. 9a, eight bundles are visible.

By 7 min after fertilization, the cones reach their maximum length and complexity. The bulk of the space within the cones is now occupied by filament bundles (Fig. 10). These bundles terminate at the plasma membrane limiting the cone, giving the irregular profile of the cone seen after 5 min. From here they extend into the cortical cytoplasm. It is difficult by thin sectioning to measure the length of an individual filament, because one can never be sure if a particular section is cut normal to the filament; however, filament bundles penetrate at least $5\text{ }\mu\text{m}$ into the cortex of the cell (see Fig. 10). In general, most of the bundles lie parallel to one another, a feature that can be substantiated by transverse sections through the cone. In these cross sections the numbers of filaments in four bundles were 17, 18, 40, and 106 (Fig. 11). From our micrographs, it seems likely that a large bundle is probably made up of a series of smaller bundles that extend into the "spikes" on the surface of the cone.

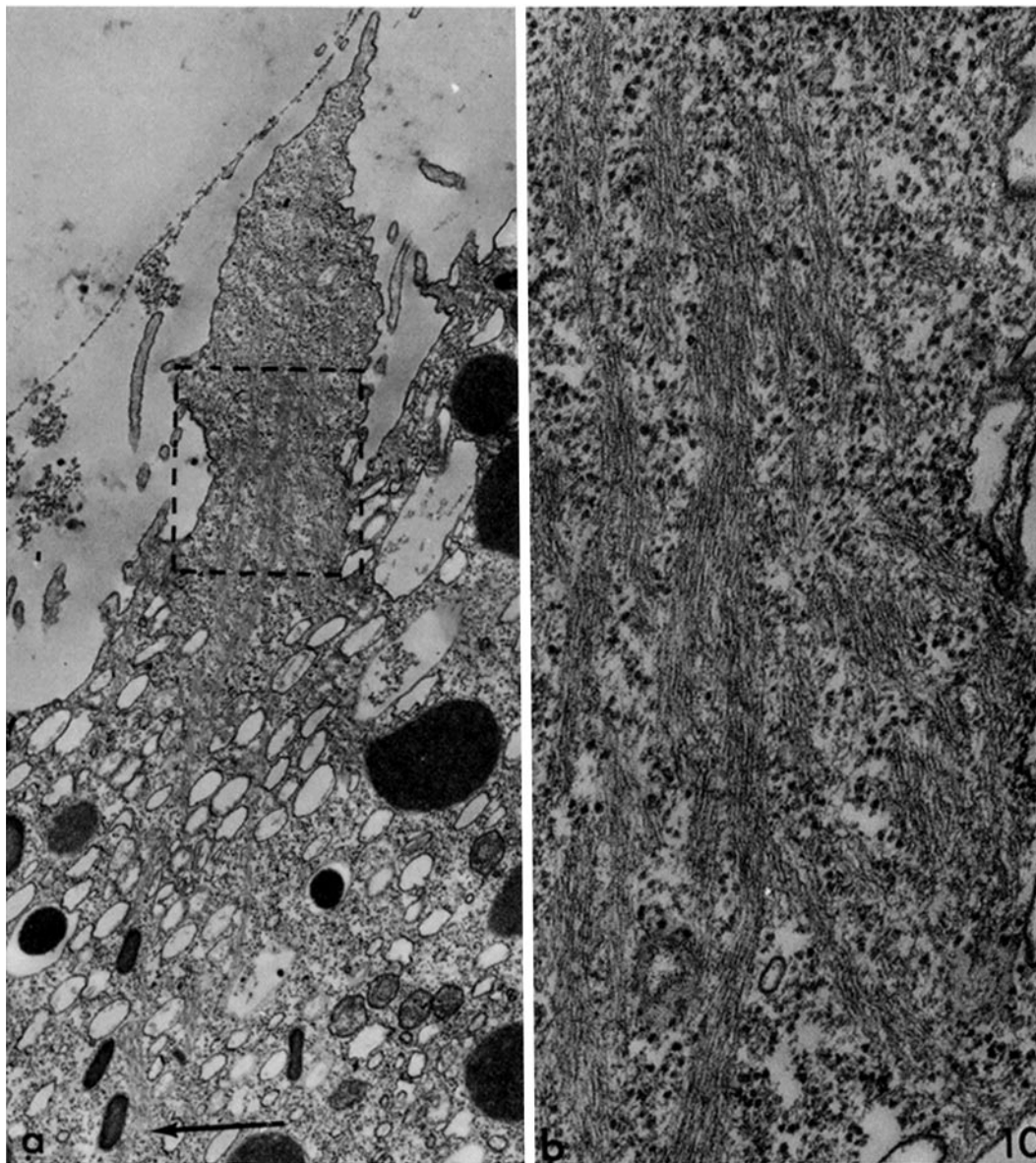


FIGURE 10 Thin section through the fertilization cone from an *Arbacia* egg fixed by procedure 2, 7 min after insemination. Note a large population of filaments (shown at higher magnification in *b*) that extends far into the medullary cytoplasm (see arrow). The area of *a* depicted in *b* is indicated by the broken lines. *a*, $\times 18,000$; *b*, $\times 74,000$.

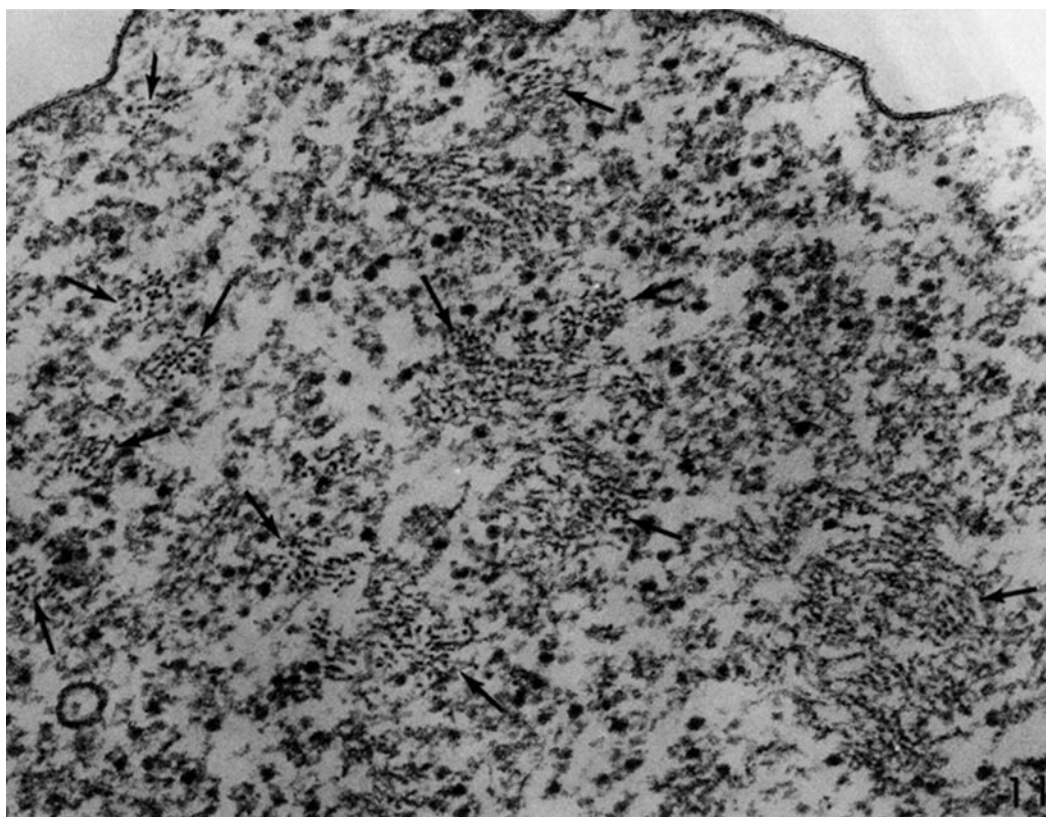


FIGURE 11 Transverse section through a portion of a cone from an *Arbacia* egg fixed by procedure 2, 7 min after insemination. The arrows indicate bundles of filaments cut in transverse or nearly transverse sections. $\times 125,000$.

By 10 min after fertilization, the cones are either the same size as after 7 min or they are reduced in size. They still have prominent filament bundles within them.

Fine-structure Observations on the Fertilization Cones and Microvilli of Oocytes at GV

The surface of an unfertilized GV oocyte is highly variable in form; in thin sections, long stretches with no surface projections can be next to a region in which we find a large number of irregular surface projections. Filaments are seen within these projections and in the egg cortex, but these are not preferentially associated with densities on the cytoplasmic face of the plasma membrane. The cortical granules can be found in the cortex or in deeper layers, depending on the stage of development of the oocyte. The vitelline layer is generally thin and discontinuous.

As has been observed previously, GV oocytes produce huge cones, and a fertilization membrane does not form (12, 19). In general, the cortex does not change after fertilization; increased numbers of microvilli do not appear, nor does the number of cortical granules decrease. However, within the fertilization cone, enormous numbers of filaments appear. The fertilization cone of an immature oocyte resembles that of a mature egg in that it lacks mitochondria, yolk granules, vesicles, and vacuoles, but instead contains large numbers of free ribosomes and bundles of filaments (12; Fig. 12). It is much larger than the cone of a fertilized mature egg, and the bundles of filaments appear to be spaced farther apart, but the orientation of the bundles and most of the filaments not being free, but rather assembled into bundles, are common to both.

Decoration of the Filaments in the Fertilization Cone with S_1

Of interest to this report is the determination of the polarity of the filaments in the cone. The major difficulty encountered is to be able to open up the plasma membrane sufficiently to enable the S_1 to penetrate into the cytoplasm and at the same time to preserve enough of the morphology of the egg surface so that a cone can be recognized. We accomplished this by immersing the eggs, 5 min after insemination (a time chosen because the cones are of about maximum size), into hypotonic medium, a procedure that tended to rupture the egg on one side, leaving the other side reasonably intact. Examination of the unruptured halves showed numerous decorated filaments in the cortex of the egg, a region that generally was $\sim 1 \mu\text{m}$ or less in thickness. These filaments often appeared in bundles. In some areas, it appeared as if these bundles were derived from the bundles of actin filaments that were formerly present within the microvilli but had been pulled in by the hypotonic treatment. However, some of the time, the microvilli remain, with a bundle of filaments in each. As reported by others (2, 3), the polarity of all the filaments in the microvilli is unidirectional, with the arrowheads pointing towards the cell center.

When we examined cones we found that the ribosomes were washed out, leaving cones of normal height, now filled only with filaments packed closely together. From images such as Fig. 13, it is clear that in every filament whose polarity could be determined, the arrowheads pointed towards the cell center. (The polarity of some could not be determined because of superposition of filaments in the section.) Individual decorated filaments can be followed for at least several microns. Approx-

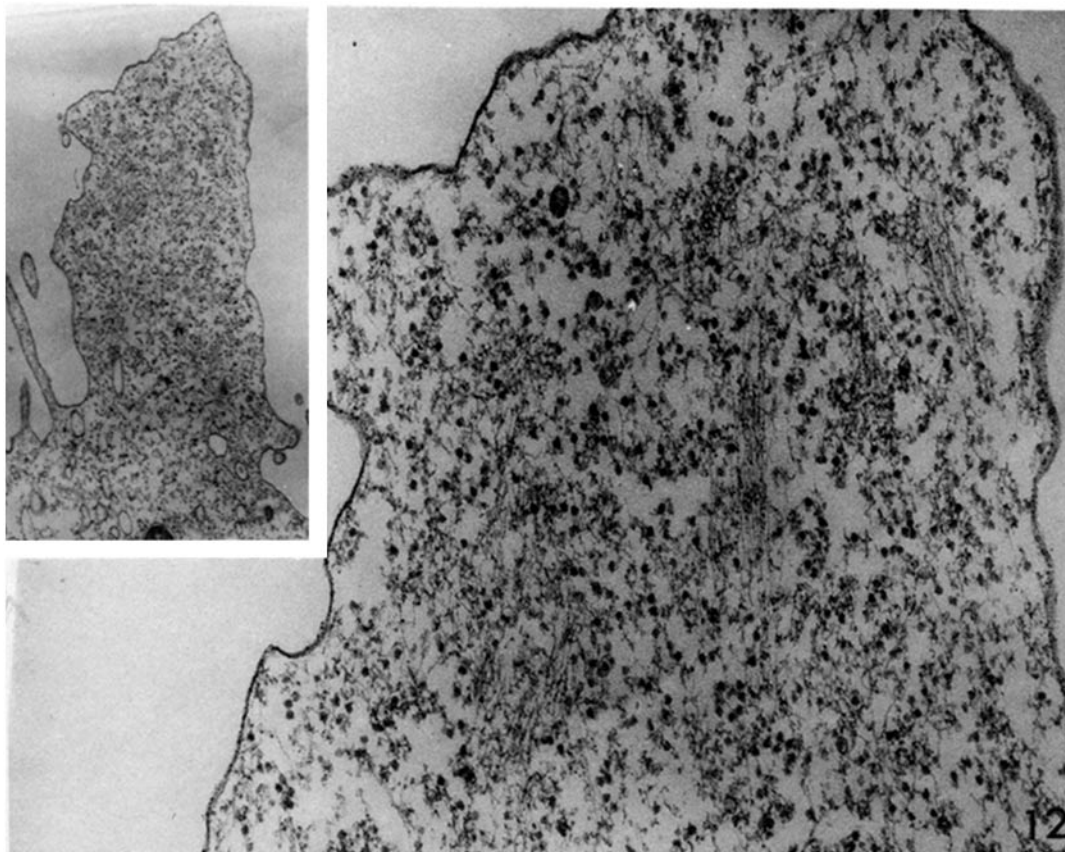


FIGURE 12 Thin section through a fertilization cone of a GV *Arbacia* oocyte fixed by procedure 1, 7 min after insemination. The inset shows the whole cone ($\times 16,000$); the rest of the figure shows a portion of it at higher magnification ($\times 70,000$). Note that bundles of filaments are present in this cone.

imately the same number of filaments is present at the midpoint of the cone as is present at the base of the filament bundles $5\ \mu\text{m}$ deep within the cortex of the cell. This suggests that the filaments we see deep within the cortex of the cell may extend into the cone proper and terminate on the plasma membrane. We have made estimates of the number of filaments present in the cone illustrated in Fig. 13. Although this value is crude, we have estimated that there are ~ 640 filaments in this cone, a spectacular number when one considers that we do not see any filaments in the egg before fertilization.

The Time-course and Amount of Acid Efflux from Arbacia Eggs after Fertilization

Because acid efflux may be related to a change in internal pH (10, 20), and because a change in internal pH may be related to the polymerization of actin at fertilization (1), we measured acid efflux from *Arbacia* eggs. Data on the time course and amount of acid efflux during fertilization of *Arbacia* have not, to our knowledge, been previously reported. A pH stat method was used (see above).

Fig. 14 shows the result of one experiment. The baseline acid efflux from unfertilized eggs was measured, then sperm were added. After a delay of ~ 30 s, acid efflux began to increase. After 2 min, the flux decreased to a plateau. These features were seen consistently in three experiments. Control experiments, in which sperm were added to already fertilized eggs or to egg water, showed that the efflux depends on fertilization and cannot be accounted for by acid produced by the sperm.

In the example shown in Fig. 14, a second increase in flux occurred at ~ 12 min postinsemination, but this was not always seen. The magnitude of the efflux per egg was measured in two experiments, and the results are summarized in Table I. Note that the timing of the rapid phase of acid efflux correlates with the time during which actin filaments are first seen in the eggs, i.e., during the first two min after insemination.

DISCUSSION

How Might Actin Filaments Produce Microvilli?

The surface of the fertilized egg changes abruptly after cortical granule exocytosis begins. Initially, i.e., 1 min after insemination, irregular protrusions, presumably left over from the exocytosis event, appear on the surface of the egg. These grow in size and complexity and, by 3 min after insemination, thin spikelike microvilli extend from many of the protrusions or from the cortex of the egg. By 5 min after insemination, many of the protrusions have been replaced by typical-looking microvilli. At the same time that these changes in microvilli are occurring, changes in the actin filaments take place. Thus, 1 min after insemination, small numbers of filaments are present in the protrusions and in the underlying cortex, and by 2 min enormous numbers of actin filaments are present. Most of these filaments are unoriented with respect to each other, except that many can be seen to contact the plasma membrane at one end. By 3 min, bundles of actin filaments can be found, although large numbers of randomly oriented filaments remain. By 5 min, bundles of actin filaments are common,

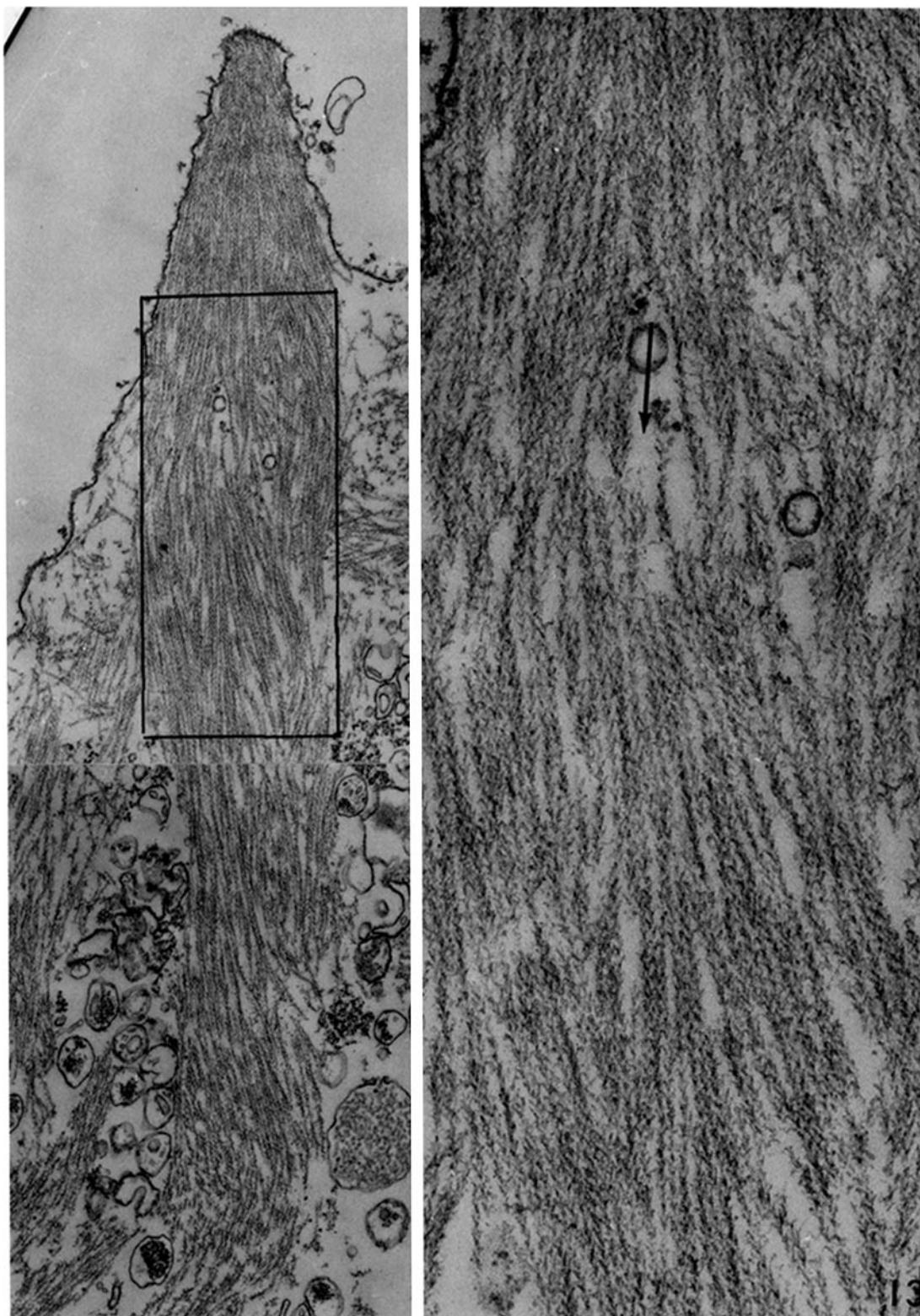


FIGURE 13 (a) Fertilization cone of an *Arbacia* egg incubated in S_1 (procedure 4), 5 min after insemination. Within this cone and extending for at least $5\ \mu\text{m}$ into the medullary cytoplasm are large numbers of decorated filaments. $\times 34,000$. (b) The portion of a indicated by the box is shown at higher magnification in b. Except in regions where superposition difficulties arise, all the arrowheads can be seen to point towards the cell center, as indicated by the arrow. $\times 95,000$.

particularly situated within the slender microvilli.

Because of these observations and because all the filaments within the microvilli have unidirectional polarity with respect to the cell surface, we propose that the filament core within each microvillus might form in the following way: The actin

filaments are first assembled from the plasma membrane, thereby assuring the requisite polarity, and, with time, adjacent filaments come together and associate laterally. As the zippering together continues, the bundles associate by lateral bridges with the membrane and, in this way, a thin microvillus grows

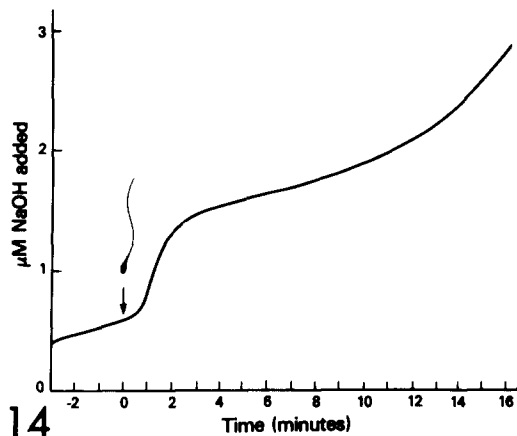


FIGURE 14 Unfertilized *Arbacia* eggs in seawater were placed in a small beaker. The suspension was stirred continuously and connected to a pH stat set to maintain the pH at 8.0, by the addition of NaOH. The unfertilized eggs release a small amount of acid. At 0 time, sperm were added; ~30 s later, increased acid efflux from the egg begins. This rapid burst of acid efflux is over by ~2 min. This is followed by a slower release of acid. Of interest is that the rapid phase of acid efflux from the egg correlates in time with the initiation of actin polymerization in the eggs.

TABLE I
Acid Efflux from *Arbacia* Eggs at Fertilization

Experiment	Unfertilized pmol/egg·min	0.5–2.5 min postinsemination pmol/egg·min	2.5–7.5 min postinsemination pmol/egg·min
A	.15	1.2	.21
B	.08	1.5	.58

out of an irregular surface projection. We have, in fact, no evidence that the actin filaments are nucleated from “magic spots” on the plasma membrane. What we do know is that at our earliest time-points one end of many of the actin filaments makes contact with the plasma membrane, and, from our study and those of others (2, 3), these filaments have unidirectional polarity with respect to the cell surface. It is, of course, possible that actin filaments or oligomers of actin in the cortical cytoplasm may associate with the plasma membrane with the requisite polarity *after* assembly or partial assembly. This alternative does not change our two-step model—both involve a membrane association or nucleation step and a second zippering step.

Such a two-step mechanism is particularly attractive because it is completely consistent with what is known about the zippering together of actin filaments in coelomocyte transformation (5). This zippering together involves a 58,000-dalton protein present not only in coelomocytes (5, 18) but also connecting the actin filaments in the microvilli of eggs (22). Thus, we can imagine that in the formation of bundles of actin filaments that contact membranes, this two-step mechanism may be commonly encountered.

The only other system in which the formation of microvilli has been studied is the reformation of microvilli in salamander intestinal epithelial cells after pressure-induced disassembly (24). In that system, the first step in microvillus formation is the reappearance of dense patches on the apical plasma membrane. This is followed by the appearance of short filaments that appear to assemble from these densities. As in the forma-

tion of microvilli in sea urchin eggs, branched microvilli or microvilli extending from a common mass of cortical cytoplasm are frequently seen. With time, these microvilli elongate to ultimately produce a brush border similar to that in untreated cells. There is one obvious difference between these two systems. In one (intestine), each microvillus appears to be nucleated from a discrete spot, and in the other (egg), association of filaments perpendicular to the membrane is at first widespread and only later becomes restricted to the apical tips of the formed microvilli. This difference might appear because in one case we are examining the formation of microvilli *de novo* (egg), and in the other we are watching the reformation of microvilli (intestine). In the latter, residual order in the membrane may persist throughout pressure treatment so that assembly can occur at one site rather than at many sites, as might be the case during the formation of microvilli in intestinal epithelial cells *de novo*. This model implies that formation of the egg microvilli *de novo* involves some fancy footwork on the part of the membrane during the zippering-together process, because initially the actin filaments that will be zippered together are attached to the membrane at points separated by fractions of a micron. Thus, as zippering continues, if our model is correct, the points of attachment to the membrane must move closer and closer together. During reformation of microvilli, on the other hand, this problem is obviated, because residual order remains in the membrane.

It would be of great interest to know what stimulates this dramatic polymerization of actin. Our acid-efflux data are consistent with existing information (1, 25), which indicates that a change in internal pH may be a regulating factor: the greatest acid efflux occurs at the same time (0.5–2.5 min postinsemination) as actin filaments first appear in the cortical cytoplasm. However, other ions (e.g., Ca^{++} or Na^{+}), as well as nonionic factors, may also function in the regulation of actin assembly (21, 23).

Actin Filaments and the Fertilization Cone

By 2 min after insemination, we find moundlike cones. By 5 min, at least in mature eggs and in many GV oocytes, these elongated cones have extending from their surfaces many spikes—the “flame-like processes” of Seifriz (19). By 7 min, these spikes are even more pronounced. Although actin filaments are common in 2- and 3-min cones, in general they are disorganized, forming bundles only infrequently. By 5 min, however, most of the filaments in the cones lie parallel to each other in bundles extending out into the microvillar-like spikes. By 7 min, the cytoplasm of the cone is largely composed of actin filament bundles. As in microvilli, the polarity of the filaments in the cones is unidirectional, with the arrowheads pointing towards the cell center. These data suggest that the actin-filament bundles of cones form in a way similar to that proposed for microvilli, that is, actin is assembled on the plasma membrane to ensure the requisite polarity, and then filaments zipper together by means of cross-bridges. With time, adjacent bundles come to be linked together, so that, by 7 min after insemination, the cone is essentially one gigantic bundle of filaments. Also, the filament bundles, with time, increase dramatically in length, so that, by 7 min after insemination, bundles of filaments extend $>5 \mu\text{m}$ into the cortical cytoplasm.

Although this model of cone assembly is similar to that discussed for microvilli, there are two striking differences between these processes. First, although the cones seem to be initially composed of discrete bundles of filaments reminiscent,

in size, of the filament bundles in microvilli, the bulk of the filament bundles remains in the single protrusion, with only small extensions into microvillar spikes. Second, the assembly of actin filaments in the cone is a local response such that in the immature oocyte this is the *only place* where actin filaments assemble, and in the mature egg the response is qualitatively and quantitatively different from the actin assembly occurring over the rest of the cortex. These differences are apparently imposed on the egg cortex by the localized contribution of the sperm, possibly by ions that the sperm contributes during fusion or by sperm-gated opening of ion channels in the egg membrane. There is evidence, in fact, for a local opening of Na^+ channels in the *Urechis* egg membrane in the immediate vicinity of the fertilizing sperm (9).

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